Generation and Characterization of Protective Antibodies to Marburg Virus

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ABSTRACT

Marburg virus (MARV) and Ebola virus (EBOV) have been a source of epidemics and outbreaks for several decades. We present here the generation and characterization of the first protective antibodies specific for wild-type MARV. Non-human primates (NHP), cynomolgus macaques, were immunized with viral-replicon particles expressing the glycoproteins (GP) of MARV (Ci67 isolate). An antibody fragment (single chain variable fragment, scFv) phage display library was built after four immunogen injections, and screened against the GP_{1-649} of MARV. Sequencing of 192 selected clones identified 18 clones with distinct V_H and V_L sequences. Four of these recombinant antibodies (R4A1, R4B11, R4G2, and R3F6) were produced in the scFv-Fc format for *in vivo* studies. Mice that were challenged with wild-type Marburg virus (Ci67 isolate) receiving 100 µg of scFv-Fc on days -1, 1 and 3 demonstrated protective efficacies ranging from 75-100%. The amino-acid sequences of the scFv-Fcs

are similar to those of their human germline counterparts, sharing an identity ranging between 68 and 100% to human germline immunoglobulin. These results demonstrate for the first time that recombinant antibodies offer protection against wild-type MARV, and suggest they may be promising candidates for further therapeutic development especially due to their human homology.

INTRODUCTION

Marburg virus (MARV), together with the five members of the *Ebolavirus* genus, constitutes the family *Filoviridae* of the order Mononegravirales. MARV causes severe and highly lethal viral hemorrhagic fevers (VHF) in both non-human primates (NHP) and humans [1]. The primary transmission of MARV is through contact with infected bodily fluids from infected humans or animals [2]. MARV was first identified in 1967 in Germany and Yugoslavia, and continues to cause sporadic outbreaks throughout equatorial Africa [3]. In the absence of a licensed vaccine or therapeutic, there are limited options beyond supportive care [4]. Although several vaccine and a few therapeutic options are currently in clinical trials for filoviruses, these are specific only to EBOV. Additionally, issues with the logistics of a complete vaccination program present a strategic gap for this global threat and do not eliminate the need for a post-exposure therapeutic program [5].

Filoviruses are nonsegmented, single-stranded negative sense RNA viruses that contain seven or more structural proteins [6]. The transmembrane glycoprotein (GP) is expressed on the viral surface and is the primary facilitating protein of entry into the host cells. The location and abundance of this protein on the virion surface makes it an attractive candidate for the development of protective antibodies. Vaccine candidates have shown varying degrees of success in animal models (Reviewed in [7]). Initial attempts focused on the use of inactivated whole virus with mixed success in NHP models while later attempts utilized virus-like replicon particles (VRP), virus-like particles (VLP), viral vectors or plasmid DNA with greater levels of protection offered [8-10]. The shared component of all these vaccine candidates was the concept of developing an immune response against GP, which would hopefully lead to the generation of protective antibodies and cellular responses.

Convalescent serum was used during the 1995 Kikwit Ebola outbreak, providing the first indication that an antibody-based therapeutic could be used to effectively treat filovirus-infected individuals. In this small study (n=8), convalescent serum treatment reduced mortality from 80% to 12.5%[11]. Since that time, there has been expanding, yet limited, success in developing protective antibody-based therapeutics against filoviruses. The recombinant anti-EBOV antibody KZ52, isolated from a human survivor, was shown to be protective in guinea pig models; however, it failed to protect in the NHP model [12, 13]. Dye *et al*. were the first to demonstrate the utility of antibody passive transfer therapies in NHP models of filovirus infections [14]. EBOV- or MARV-infected NHPs were fully protected when treated with immunoglobulin G purified from species-matched convalescent serum, even when treatment was delayed 48 hours post-infection. The first utilization of a monoclonal therapy for MARV has been recently reported by Fusco *et al*. where they found two mAbs which bind to the GP2 subunit which were able to provide protection, but to a mouse-adapted Ravn strain of Marburg virus (RAVV) [15].

In this study we present the generation, isolation and characterization of a series of macaque, high-affinity single chain variable fragments (scFvs) targeting MARV GP as well as the protection in a mouse model obtained by these antibodies in the scFv-Fc (Yumab).

RESULTS

Macaque Immunization and Antibody Generation

A single cynomolgus macaque was intramuscularly (i.m.) immunized with four sequential injections of virus replicon particles (VRP) expressing the Marburg GP (isolate Ci67) at surface of cells following viral replication of the complex. The macaque developed increasing anti-GP Ab titers as evaluated by ELISA with a titer of 1:12800 after the second boost and 1:50,000 following the third. The final boost was given three months after the third injection and eight days later bone marrow samples were harvested. Bone marrow samples were taken on days 3, 6, 8, 12, 18 and 21. The strongest DNA amplification was observed at the day eight time point (Supplemental Figure 1 and supplemental Table S 1-3) before the quantity of the amplified variable gene products decreased. The

amplified products of VH1 through VH9 and VL1 through VL7 were combined from day eight collections and cloned into pGemT for the respective construction of κ light chains and Fd sub-libraries.

Library construction and Isolation of scFvs specific to MARV-GP

The library underwent a multi-step panning against the Ci67 isolate of MARV GP_{1-649}. Four successive wash rounds at 5, 10, 20, and 40 washes isolated 3 x 10^7 phage. Eighteen clones with distinct V_H and V_L sequences were isolated from 194 clones that were sequenced following the panning with MARV GP_{1-649}. (Table 1) The overall identity of the macaque V_H and V_L sequences with their human genetic counterparts averaged 76.7% for V_H and 82.1% for V_L.

Table 1. Germline sequence similarity of 18 distinct scFv antibody fragments isolated from panning. Macaque V_H and V_L similarity with their human germline counterparts were calculated.

	Heavy Chain (V_H)		Light Chain (V_L)	
Antibody	Human Germline family ID	% Identity	Human Germline family ID	% Identity
R4A1	IGHV3-11*04	88.8	IGKV1-5*01	93.6
R4B11	IGHV1-69*09	80.6	IGKV1D-12*01	88.4
R4G2	IGHV3-23*01	60.8	IGKV1-5*01	78.9
R4G7	IGHV3-23*01	77.3	IGKV1-5*01	78.9
R4G8	IGHV3-23*02	76.3	IGKV1-5*01	81.1
R4G9	IGHV3-23*01	79.4	IGKV1-5*01	81.1
R4G10	IGHV3-23*04	61.9	IGKV1-5*01	78.9
R4G11	IGHV3-23*02	75.3	IGKV1-5*01	81.8
R4H11	IGHV3-23*02	75.3	IGKV1-5*01	81.1
R4H12	IGHV3-23*02	76.3	IGKV1-5*01	81.1
R3C4	IGHV1-69*09	84.7	IGKV1D-12*01	85.3
R3D4	IGHV3-23*02	75.3	IGKV1-5*01	81.1
R3F6	IGHV3-23*01	78.4	IGKV1-5*01	80.0
R3G2	IGHV3-23*02	71.1	IGKV3-15*01	80.0
R3G5	IGHV4-b*02	75.8	IGKV1-39*01	85.3
R3H2	IGHV3-23*02	77.3	IGKV1-5*01	81.1
R3H6	IGHV3-23*02	77.3	IGKV1-5*01	80.0
R3H10	IGHV3-23*01	89.7	IGKV1-5*01	80.0

Antibody recovery and characterization

Each of the 18 distinct scFv recovered from the library was assessed for its binding capacity with MARV Ci67 GP_{1-649} by surface plasmon resonance (SPR). (Table 2) The affinities of the anti-MARV scFv were evaluated under standard conditions, using 800 second elution steps in HBS-EP buffer (Figure 1), and resulting values ranged from 155 nM for R3H2 to 0.14 nM for R3F6 (Table 2). Of note, R3G5 was unable to produce sufficient quantities of antibody to be further tested.

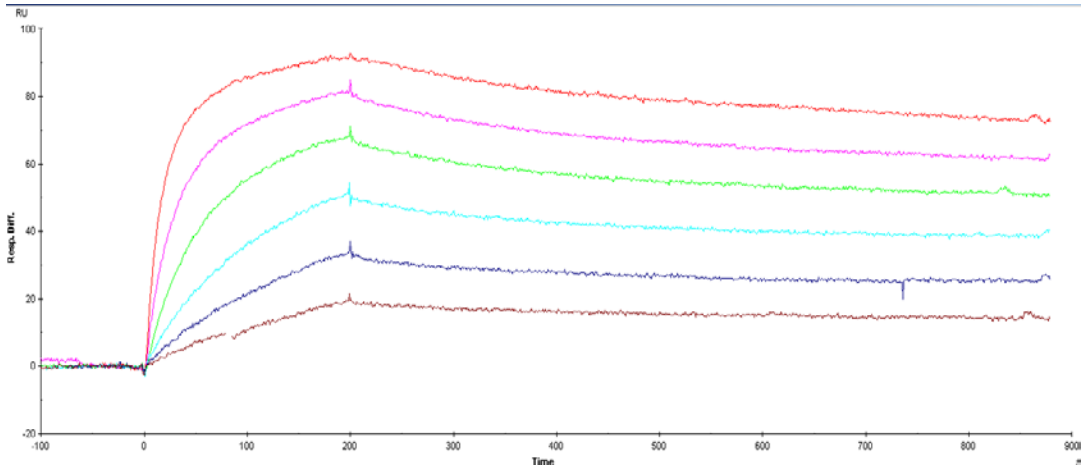


Figure 1: Representative BiaCore sensorgram of anti-MARV antibody R4A1. R4A1 affinity was measured at 4.4 nM against MARV GP, utilizing an 800 second elution.

Classical epitope determination methods utilizing peptide arrays were not successful. To characterize these antibodies, we determined competition groups and isolated specific epitopic families within the 17 remaining antibodies using BiaCore analysis. SM5 chips were coated with GP_{1-649} and were pairwise added for sequentially assessment by BiaCore. As an example, competitive antibodies had no change in signal (Figure 3, blue line) while antibodies biding to a new epitope had an increased signal above the first set. (Figure 2 red line). Using this analysis, we identified three distinct groupings of antibodies which were non-competitive (Table 2). The antibody R3C4 is not determined (ND) because its dissociation rate was too rapid to determine a competition grouping.

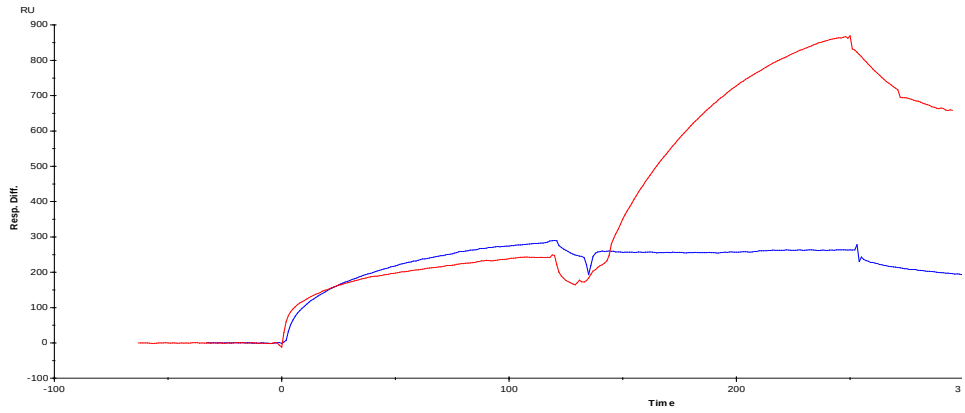


Figure 2: BiaCore sensorgram of binding analysis between two competitive antibodies (blue) and two non-competitive antibodies (red).

Table 2. Antibody Affinities as measured by Bia-Core with Epitope groping

Antibody Fragment	k_{on} $M^{-1}s^{-1}$	k_{off} s^{-1}	K_D (nM)	Epitope Group
R4A1	6.42×10^{4}	2.85×10^{-4}	4.4	2
R4B11	8.76×10^{4}	4.07×10^{-4}	4.6	1
R4G2	1.19×10^{4}	4.54×10^{-4}	38	2
R4G7	8.19×10^{4}	3.79×10^{-4}	4.6	1
R4G8	9.23×10^{4}	6.3×10^{-3}	68	3
R4G9	3.1×10^{4}	1.75×10^{-4}	5.6	3
R4G10	1.39×10^{4}	7.46×10^{-4}	54	2
R4G11	7.5×10^{4}	4.75×10^{-4}	6.3	2
R4H11	3.75×10^{4}	4.17×10^{-4}	11	1
R4H12	1.46×10^{5}	5.65×10^{-4}	3.9	2
R3C4	1.16×10^{4}	3.86×10^{-4}	33	ND
R3D4	1.75×10^{4}	8.98×10^{-4}	5.1	1
R3F6	1.99×10^{5}	2.35×10^{-5}	0.14	1
R3G2	8.3×10^{3}	5.78×10^{-4}	69.7	2
R3H2	6.39×10^{3}	9.93×10^{-4}	155.0	2
R3H6	1.74×10^{4}	5.61×10^{-4}	32.3	2
R3H10	4.13×10^{4}	2.83×10^{-4}	6.9	2

Four antibodies from the groups above were chosen based on their high affinity to GP_{1-649}, sequence homology and growth characteristics (data not shown). These antibodies were produced in the scFv-Fc format to assess for cross binding by western blot analysis as well as for use in protection studies. The selected antibodies were assessed by western blot analysis; demonstrating cross reactivity to the MARV isolates Ci67, Musoke and Angola, but with no reactivity to Ravn virus (Figure 3). Of the four antibodies assessed, all bound strongly to Ci67; R4A1, R4B11 and R3F6 bound strongly to Musoke; and only one of the antibodies, R3F6, demonstrated moderate binding to the Angola isolate.

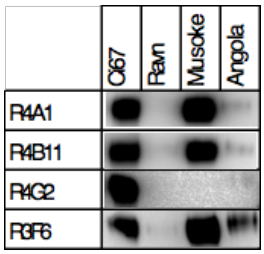


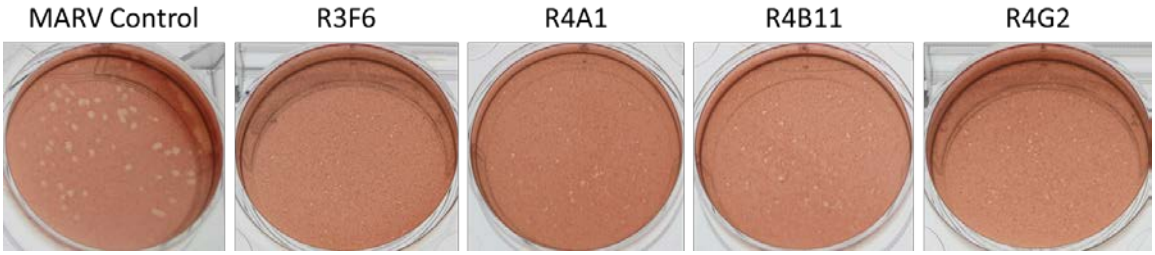
Figure 3: Western blots analysis of the reactivity of the scFv-Fc antibodies to whole irradiated virus. In each 4-12% gradient gel, each lane was loaded with 3uL of a 1:500 dilution of sucrose purified Marburg virus isolates corresponding to the labeled well.

In vitro antibody neutralization

The identification of neutralizing mAbs to MARV has been problematic, with no known neutralizers reported in the literature, although Kajihara *et al*. was able to demonstrate an inhibitory mechanism specific to viral budding [16]. In this study we utilized two separate assays to evaluate for neutralization to MARV. ScFv's for 17 of the antibodies (R3C4 was not assessed due to low expression) were tested in a VSV pseudovirion assay expressing the Musoke variant of GP as well as the classical plaque reduction neutralization test (PRNT) assay utilizing Ci67 isolate of wild-type MARV. In both of these assays, no antibody reached a PRNT titer of 80% inhibitory concentration (Supplemental Figure 2). In the classical neutralization assay which measures the ability of a molecule to block viral entry to the cell, plaque sizes appeared as smaller "pinpoint" plaques and often took an extra day to detect but failed to reach a PRNT titer of 80%. (Figure 4a) The reduction of these plaque

sizes were significant for four of the antibody fragments tested, R3F6, R4A1, R4B11, and R4G2. (Figure 4)

(a)



(b)

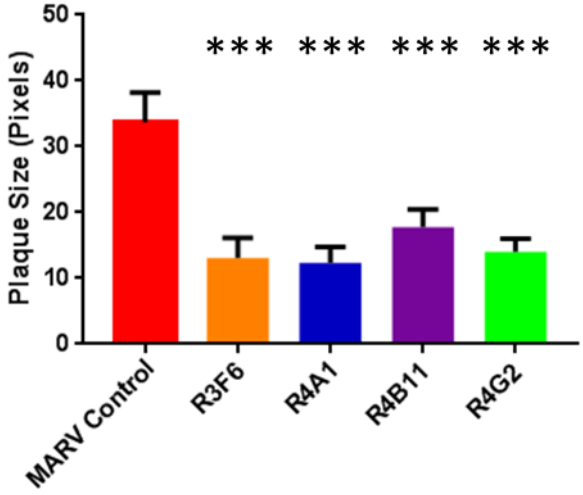


Figure 4: (A) Photographic representation of viral plaques and (B) corresponding sizes in Vero E6 cells. ***All plaque sizes were highly significant to a p-value <0.0001 by utilizing a two-tailed t-test for the four antibody fragments tested against control virus.

In vivo mouse protection study

To investigate the *in vivo* protection of the four selected candidates (R4A1, R4B11, R4G2 and R3F6), each of them was reformatted as scFv-Fc (Yumab) and tested in INF α/β receptor knockout mice (IFNAR-/-) challenged with wild-type MARV Ci67. Standard mouse models, using C57BL/6 or BALB/c, could only be utilized with a mouse adapted variant of MARV Ravn as previously done by others[17]. These antibodies bind to Ci67 with some cross reactivity to Angola or Musoke but are not cross reactive to Ravn. All scFv-Fc antibodies were protective against a lethal MARV Ci67 challenge of 1000 PFU (Figure 5). The antibody R3F6 demonstrated the best efficacy with 100% protection, under the tested conditions, while R4A1, R4B11, and R4G2 had protective efficacies of 75%, 80%, and 85%, respectively. Thirty-five days after the challenge, surviving mice were re-infected with a

second injection of 1000 PFU by i.p. with no antibody treatment. All mice survived after the second challenge with no loss in weight, demonstrating that these mice were able to develop a protective memory immune response. (Supplementary Figure)

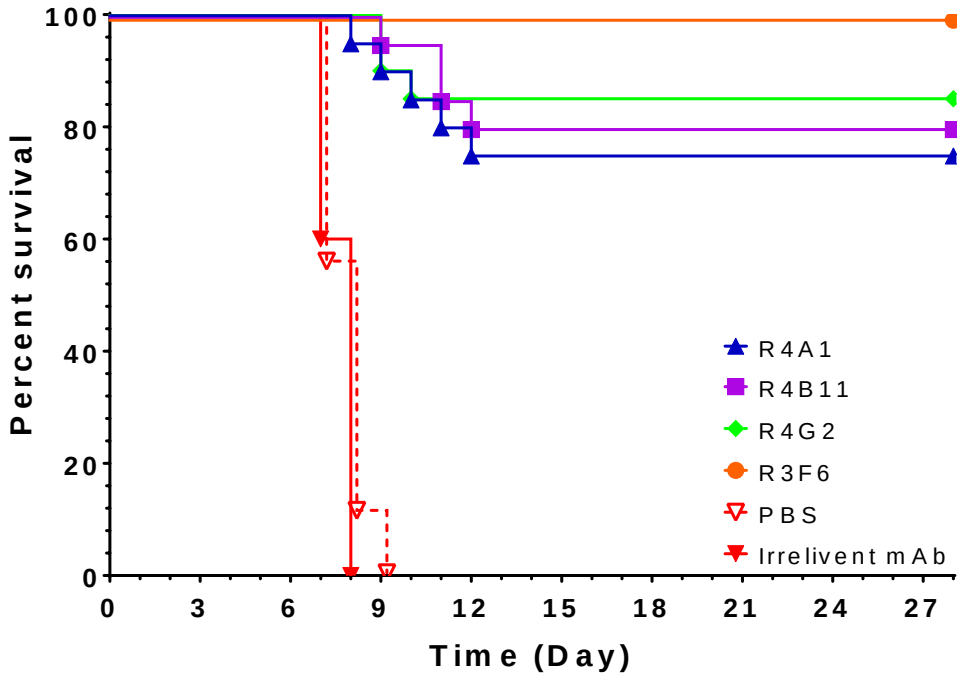


Figure 5: Percent survival of IFNAR-/- mice administered MARV GP specific antibodies. Each mouse was administered 100ug of antibody treatment, irrelevant antibody (n=10), or PBS (n=10) on Days -1, 1, and 3. Mice were challenged with 1000 pfu MARV Ci67 on D0.

DISCUSSION

Previous studies have demonstrated that post-exposure polyclonal antibodies as well as recombinant monoclonal antibodies provide protection against filoviruses in NHP models. Although there is no clear path for the down selection of antibodies, we chose an approach which identified high binding affinity to the antigen, sequence homology, production capacity and finally protection. Previous work has identified that neutralization may not be the result of higher affinity [18]. This gap in the understanding initial *in vitro* characteristics to protection could be one reason that few monoclonal antibodies have advanced to protection studies. To date no reports identify protection against wild-type MARV in the literature. Previous studies have proposed that the protection of MARV may proceed by a mechanism other than the classical mechanism which blocks virus entry [14]. It has

recently been shown that antibodies can inhibit the virus by a separate mechanism, viral budding [16]. In this study we present the first monoclonal antibodies developed from NHP immune libraries providing protection in an animal model for MARV.

Although having both vaccines and pre-exposure therapeutics against viral hemorrhagic fevers would be optimal, the reality is that viral diseases can occur in areas that were not previously known to have a history of that disease or strain so that a vaccination campaign would not be easy to implement. This occurred in 2014 with the EBOV outbreak in western Africa, demonstrating that the emergence of a virus could present itself in a population unvaccinated or prepared for such an insult of disease. Given the half-life of current human antibodies, a pre-treatment of a known protective antibody or cocktail could be administered at least to health care workers, first-responders, or service members prior to an operation. This prophylactic treatment would provide sufficient protection for several weeks to months, with titers possibly higher than those provided by a rapid vaccine programs. Antibodies could thus represent effective pre- and post-exposure molecules.

In these studies, we report the first *in vivo* protective recombinant antibodies against wild type MARV. We believe these antibodies are promising candidates for use in the development of an antibody cocktail for therapeutic applications.

MATERIALS AND METHODS

Macaque immunization

Virus replicon particles (VRPs) on a Venezuelan equine encephalitis virus platform were first developed by Pushko *et al*. [19]. Filovirus-specific VRPs expressing MARV GP at their surface have been previously shown protection in rodents and NHPs [20]. VRPs expressing MARV GP were injected intramuscularly (i.m) into a cynomolgus macaque (*Macaca fascicularis*). The first injection consisted of MARV VRP at a concentration of $9.0x10^8$ VRP/mL. Two additional injections were completed at 30 day intervals followed by a final booster (fourth) injection 88 days after the third injection, all at $9.0x10^8$ VRP/mL.

The macaque immunizations were approved by the Institut de Recherche Biomédicale des Armées Ethics committee (Comité d'éthique de l'Institut de Recherche Biomédicale du Service de Santé des Armées) under authorization no. 2008/03.0 and were performed in accordance with all relevant French laws and ethical guidelines, including, in particular (1) "partie règlementaire du livre II du code rural (Titre I, chapitre IV, section 5, sous-section 3: expérimentation sur l'animal)," (2) "décret 87-848 du 19-10/1987 relatif aux expériences pratiquées sur les animaux vertébrés modifié par le décret 2001/464 du 29/05/2001," (3) "arrêté du 29 octobre 1990 relatif aux conditions de l'expérimentation animale pour le Ministère de la Défense," and (4) "instruction 844/DEF/DCSSA/AST/VET du 9 avril 1991 relative aux conditions de réalisation de l'expérimentation animale." Animal care procedures complied with the regulations detailed under the Animal Welfare Act and in the Guide for the Care and Use of Laboratory Animals . Animals were kept at a constant temperature (22 °C ± 2 °C) and relative humidity (50%), with 12 h of artificial light per day. Animals were anesthetized before the collection of blood or bone marrow by an intramuscular injection of 10 mg/kg ketamine (Imalgene®, Merial). If the animal technicians suspected that the animal was in pain, on the basis of their observations of animal behavior, analgesics were subsequently administered, through a single intramuscular injection of 5 mg/kg flunixine (Finadyne®, Schering Plough) in the days after interventions.

Construction and screening of the anti-MARV antibody gene library

RNA from lymphocytes of the macaque bone marrow was prepared with Tri Reagent (Molecular Research Center Inc, Cincinnati, USA). The isolated RNA was reverse transcribed to cDNA using Superscript II and oligo (dT) (Invitrogen, USA). Combinations of forward and reverse primers were used to amplify the regions coding for the variable regions VLκ and VH as previously described [21]. PCR products were cloned in the pGemT vector (Promega, Madison, Wisconsin) according to the manufacturer's instructions, yielding two sub-libraries encoding the heavy chains (Fd fragment) or the kappa light chains.

The pGemT cloned PCR products were reamplified using two macaque oligonucleotide primer sets to introduce restriction sites for library cloning as described before [22-24]. In brief, the secondary PCRs

were carried out for each forward oligonucleotide primers separately to keep the diversity. Each PCR was performed in a volume of 100µl using 100ng purified PCR reaction product of the pGemT cloned cDNA, 2.5 U Go Taq polymerase (Promega, Mannheim, Germany), 200 µM dNTPs each and 200 nM of each oligonucleotide primer for 20 cycles (30 s 94°C, 30 s 57°C, 30 s 72°C), followed by 10 min 72°C. The PCR products were separated by 1.5% (w/v) agarose gel, cut out and purified using Nucleospin Extract II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

The construction of the library was completed in two subsequent steps. First, the PCR products encoding VL were cloned into pHAL35. pHAL35 was derived from pHAL14 with an additional *Sfi*I site for VH cloning and Myc-His tag orientation instead of His-Myc [25]. Second, the VH PCR fragments were cloned. A total of 5 µg pHAL35 and 2 µg VL were digested using 50 U *Mlu*I and 50 U *Not*I (NEB, Frankfurt, Germany) in a 100 µl reaction volume for 2 h at 37°C. Afterwards, 0.5 U calf intestinal phosphatase (MBI Fermentas) was added and incubated for further 30 min. This dephosphorylation step was repeated once. The vector was purified using the Nucleospin Extract II Kit. 270 ng VL were cloned into 1 µg of the dephosporylated pHAL35 using 1 U ligase (Promega, Mannheim, Germany) overnight at 16°C. The ligation reactions were precipitated with ethanol and sodium acetate and the pellet was washed twice with 70% ethanol. These reactions were electroporated (1.7 kV) in 25 µl XL1-Blue MRF' (Agilent, Böblingen, Germany). The transformed bacteria were plated onto 2xYT agar plates (Sambrook and Russell, 2001) (25 cm petri dishes) supplemented with 100 µg/mL ampicillin, 20 µg/mL tetracycline and 100 mM glucose. The colonies were harvested by suspending in 40 mL 2xYT media with a Drigalsky spatula. Plasmids were isolated using the Nucleobond Plasmid Midi Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Afterwards, 5 µg of each VL chain library as well as 2 µg of the VH fragments were digested using 50 U *Hind*III (NEB) in a 100 µl reaction volume overnight at 37°C followed by 50 U *Sfi*I (NEB) for 2.5 h at 50°C. In total 4 transformations were performed and pooled. The harvested bacteria representing the final antibody gene libraries were aliquoted and stored at -80°C.

Library packaging

400 mL 2xYT medium supplemented with 100 µg/mL ampicillin and 100 mM glucose were inoculated with the library glycerin stock of the pooled library [26]. The bacteria were grown to $O.D._{600}$=0.4 - 0.5 at 37°C and 250 rpm. 25 mL bacteria (~$1.25x10^{10}$ bacteria) were infected with $2.5x10^{11}$ Hyperphage, incubated at 37°C for 30 min without shaking, followed by 30 min at 250 rpm [27, 28]. The infected cells were harvested by centrifugation for 10 min at 3220 xg and the pellet was resuspended in 30 mL 2xYT supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin, and cultivated over night at 30°C and 250 rpm. Bacteria cells were pelleted for 10 min at 10000 xg. Phage particles in the supernatant were precipitated with 1/5 volume of 20% PEG/2.5 M NaCl solution for 1 h on ice with gentle shaking and pelleted 1 h at 10000 xg at 4°C. The precipitated phage were re-suspended in 10 mL phage dilution buffer (10 mM TrisHCl pH7.5, 20 mM NaCl, 2 mM EDTA), sterile filtered using a 0.45 µm filter and precipitated again with 1/5 volume of PEG solution for 20 min on ice, and pelleted 30 min at 10000 xg at 4°C. The precipitated phage were re-suspended in 300 µL PBS (phosphate buffered saline) and cell debris was pelleted by additional centrifugation for 5 min at 15400 xg at 20°C. The supernatant containing the scFv phage were stored at 4°C. The library packaging was analyzed by SDS-PAGE, Western blot and anti-pIII immunostaining as described before [18].

Screening of the library was performed as described elsewhere, [29] except that 5, 10, 20, and 40 washes were performed for each successive round of panning. MARV GP_{1-649} was utilized as the antigen and TBS-Tween 20 0.1% as the washing buffer.

Sequence Analysis

A sequence analysis of similarities between macaque V_H and V_L and the closest human germline genes encoding V_H and V_L was performed utilizing the IMGT database (http://www.imgt.org/IMGTlect/) (Table 1). Additionally, the degrees of identities between the macaque V regions with their most similar human germline counterparts were calculated with DomainGapAlign (http://www.imgt.org/3Dstructure-DB/cgi/DomainDisplay.cgi) [29].

Affinity measurements

Affinities were measured by surface plasmon resonance (SPR) utilizing a BIAcore-3000 instrument (Biacore, Uppsala, Sweden). The MARV GP1-649 was immobilized at a maximum of 1000 RU on a CM5 chip (BiaCore) via amine coupling according to the manufacturer's instructions. A 30 µL/min flow rate was maintained for the measurement. For each scFv, eight dilutions were prepared in HBS-EP buffer (Biacore) with elution times greater than 1000 seconds. Following each dilution, the chip was regenerated with 1.5M glycine buffer (Biacore) run at 10 µL/min for 50 seconds. For competition BiaCore epitope binding, MARV $GP_{1\text{-}649}$ was immobilized at a maximum of 400 RU on a CM5 chip (BiaCore) as above. Sets of two antibodies were injected in tandem with the second antibody injection just after the maximal saturation of the epitope. Following the second antibody injection, the chip was regenerated with 1.5M glycine buffer (Biacore) run at 10 µL/min for 50 seconds This process was completed until all antibodies could be assessed with one another.

ScFv-Fc production and purification

ScFv fragments isolated by antibody-phage display were subcloned into pCSE2.5-mIgG2c-Fc-XP and produced as scFv-Fc (Yumab) in HEK293-6E cells (National Research Council (NRC), Biotechnological Research Institute (BRI), Montreal, Canada) cultured in chemically defined medium F17 (Invitrogen, Life Technologies, Darmstadt, Germany) supplemented with 1 g/L pluronic F68 (Applichem, Darmstadt, Germany), 4 mM L-glutamine (GE Healthcare, Freiburg, Germany) and 25 mg/L G418 (GE Healthcare, Freiburg, Germany), as previously described [30]. The Fc was of murine origin and the scFv-Fc format is equivalent to IgG. For the scFv-Fc production, DNA was used for the transient transfection of 25 mL cultures of HEK293-6E cells in 125 mL Erlenmeyer shake flasks. After 48 hours of culture with shaking at 110 rpm in a Minitron orbital shaker (Infors, Bottmingen, Switzerland) at 37 °C, under an atmosphere containing 5% CO_2, one volume of culture medium, with a final concentration of 0.5% (w/v) tryptone N1 (TN1, Organotechnie S.A.S., La Courneuve, France) was used for the purification of a scFv, whereas scFv-Fc were purified on a UNOsphere SUPrA

column (Biorad, Munich, Germany) with a Profinia apparatus (Biorad, Hercules, California, USA), according to the manufacturer's instructions.

Cell Based Neutralization Assay

Antibody samples, in the scFv format, were titrated in complete MEM supplemented with 10% FBS. Antibody dilutions were added, in decreasing dilutions, to a constant viral titer for 65 PFU per well for a 1 hr incubation at 37°C. Dilutions were plated in triplicate on 6-well plates containing 95-98% confluent Vero E6 cells. After a 1 hr incubation at 37°C, wells were overlaid with 1% agarose in Eagle's Basal medium (EBME) with 10% FBS and 0.1% gentamicin and returned to the incubator for 7 days. On day 7, a 1% agarose secondary overlay containing 4% neutral red was added and after 1 more day at 37 °C, plaques were counted [31].

Western Blot Analysis

Irradiated MARV antigen (Ci67, Ravn, Angola, and Musoke) was mixed with 4X loading buffer (Life Technologies) and 2-betamercaptoethanol (BioRad). The samples were heated at 70C for 10 minutes and 10ul was loaded on 4-12% Bis-Tris precast gels (Life Technologies). 10ul of precision plus protein dual color standard (BioRad) was added to the gels as well. The gels were run at 150V for 90 minutes in 1X MOPS running buffer (Life Technologies). The gels were transferred to nitrocellulose membranes (Life Technologies) via the IBLOT. The membranes were blocked with 5% milk (Microbiology) in PBS (Sigma) plus 0.02% Tween20 (Sigma Aldrich) (PBST) for 2hrs at RT on shaker. The primary antibodies were added at 1ug/ml in 10ml of blocking buffer and incubated for 1hr at RT on shaker. The membranes were washed 3X with PBST at 10 minutes each. Secondary antibody horseradish peroxidase goat anti-mouse gamma was added at 1:5000 in blocking buffer for 1hr at RT on shaker. The membranes were washed 3X with 10ml of PBST for 10 minutes. Gels were imaged on BioRad imager after staining with TMB (Life Technologies).

Murine protection study

Research at U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) was conducted under an Institutional Animal Care and Use Committee (IACUC) approved protocol in

compliance with the Animal Welfare Act, PHS Policy, and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Associa-tion for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Specific pathogen-free 6- to 8-week-old male and female INF α/β receptor knockout (IFNAR -/-) mice were utilized (Jackson Laboratory Bar Harbor, ME) as a model for filovirus infection. Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. One hundred micrograms of each antibody was administered intraperitoneally (i.p.) to groups of mice (n=10/gender with n=20/treatment group) as a scFv-Fc fusion, on Days -1, 1, and 3. On Day 0, mice were transferred to a Biosafety Level 4 containment area and challenged by i.p. inoculation utilizing 1000 plaque forming units (PFU) MARV Ci67. Mice were weighed and monitored and once or twice daily upon onset of symptoms for 28 days post infection.

SUPPLEMENTAL METHODS:

Viral pseudotypes bearing MARV Musoke GP were generated by infecting 293T cells expressing MARV Musoke GP with VSVΔG, as described previously [32]. Vero cells were maintained at 37°C and 5% CO2 in high-glucose Dulbecco's modified Eagle medium (DMEM) (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum. For antibody neutralization experiments, pre-titrated amounts of pseudotype VSV-MARV particles (MOI ≈ 1 IU per cell) were incubated with increasing concentrations of test antibody or scFv molecule, starting at 350 nM concentration, at room temp for 1 h, prior to addition to cell monolayers in 96-well plates. Viral infectivities were measured

by automated enumeration of eGFP+ cells (infectious units; IU) using a CellInsight CX5 imager (Thermo Fisher) at 12–14 h post-infection. Viral neutralization data were subjected to nonlinear regression analysis to extract EC50 values (4-parameter, variable slope sigmoidal dose-response equation; GraphPad Prism).

Disclamer:

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

SUPPLEMENTAL DATA:

Table S-1. ELISA serum titer response following each successive dose of VRP

n	1	2	3	4
Date	0	32	62	150
Dose	2 x 425 µL at $0.9x10^9$ VRP/mL	2 x 410 µL at $0.9x10^9$ VRP/mL	2 x 400 µL at $0.9x10^9$ VRP/mL	2 x 400 µL at $0.9x10^9$ VRP/mL
ELISA Titer (GP)	NT	>1/316,000	1/500,000	NT
ELISA Titer (Live Virus)	NT	>1/316,000	1/450,000	NT

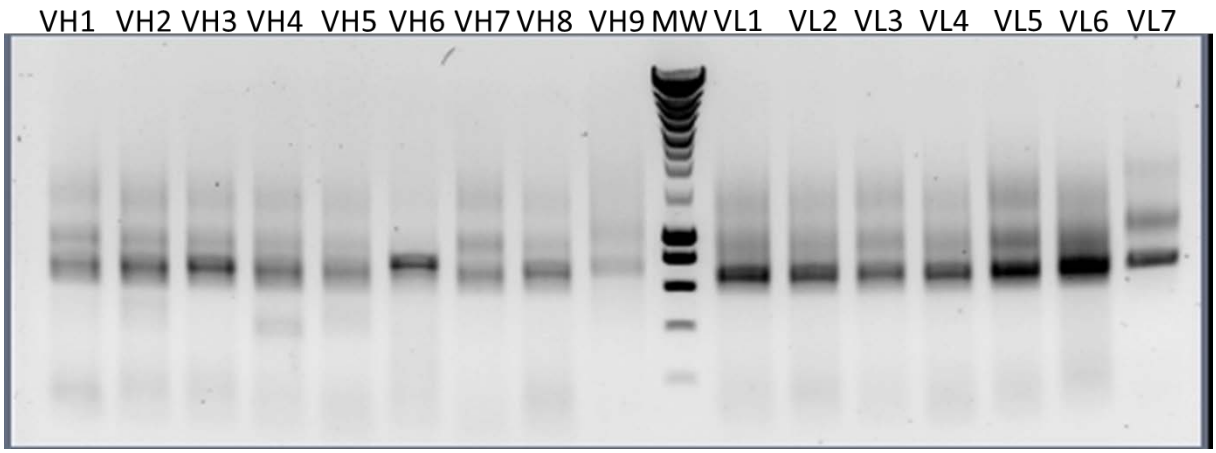
Table S-2: RNA Extraction quantities following successive bone marrow sampling

Days from Start	149	153	156	158	162	168	171
Day of Sampling	Pre-boost	D3	D6	D8	D12	D18	D21
RNA Isolated*	0.25 mg	0.167 mg	0.258 mg	0.225 mg	0.338 mg	0.200 mg	0.189 mg

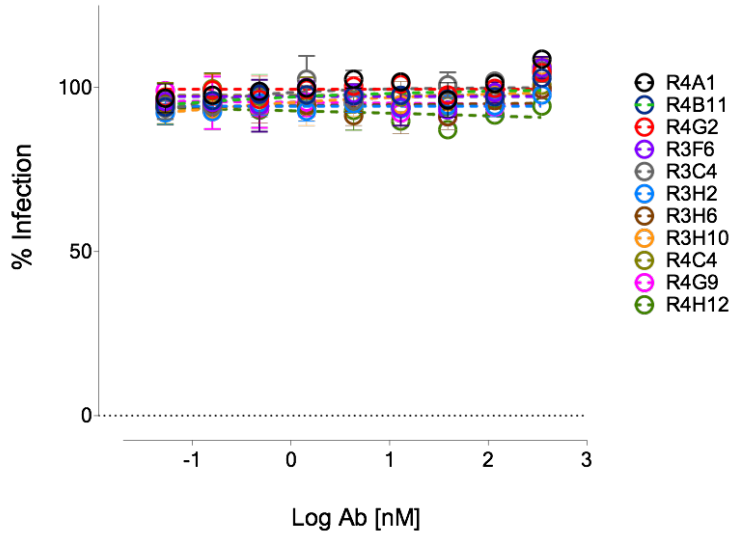
*RNA samples are reflected as solid recovery. Generally, each sample is in 200 µL of ddH2O

Table S-3: Heavy and light chain amplification by RT-PCR

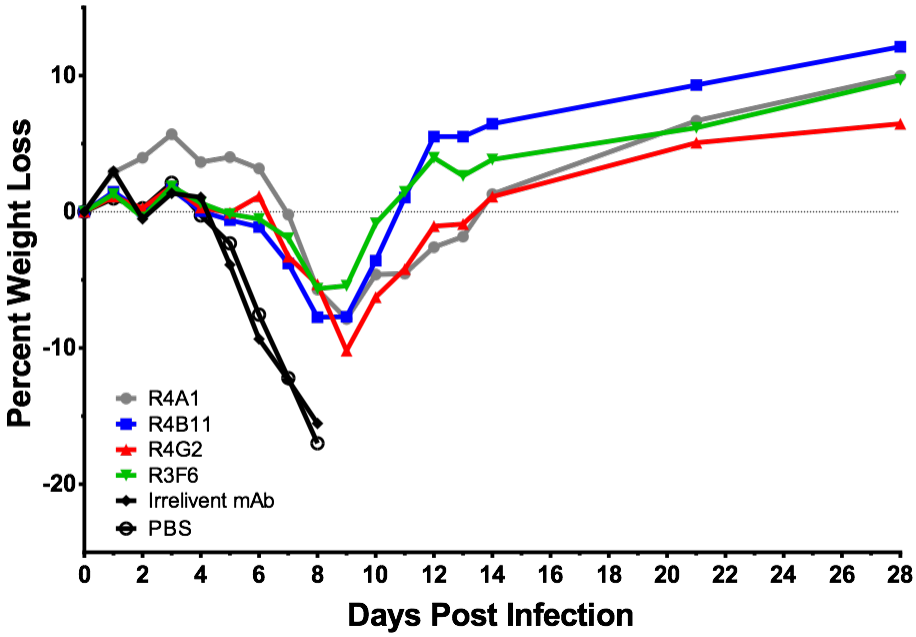
Date from Start	149	153	156	158	162	168
Day of Sampling	Pre-Boost	D3	D6	D8	D12	D18
Lk amplified families (- to +++)						
Vk1	-	-	++	+++	+++	+++
Vk2	-	+	++	+++	+++	+++
Vk3	-	-	++	+++	+++	++
Vk4	-	-	++	+++	+++	+++
Vk5	-	-	++	+++	++	+++
Vk6	+	+	++	+++	+++	+++
Vk7	+	+	++	+++	+++	+++
H amplified families (- to +++)						
VH1	+	+	++	+++	++	+
VH2	-	+	+	+++	+	+
VH3	-	+	+	+++	++	++
VH4	-	-	++	++	++	++
VH5	-	-	-	+++	+	+
VH6	-	-	-	+++	+	+
VH7	+	+	+	+++	++	+
VH8	+	+	+	+++	++	++
VH9	-	-	-	++	+	+



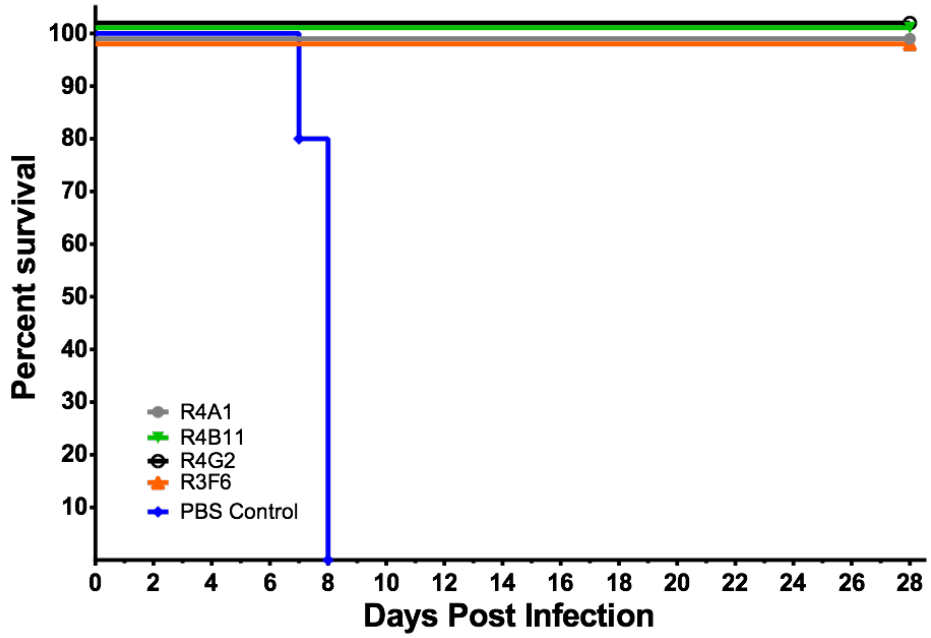
Supplemental Figure 1: Amplification of V_H and V_L chains on the eight day following the final injection. Lanes 1-9 show the nine amplified V_H families, lane 10 is the molecular weight marker, and lanes 11-17 show the seven amplified V_L families.



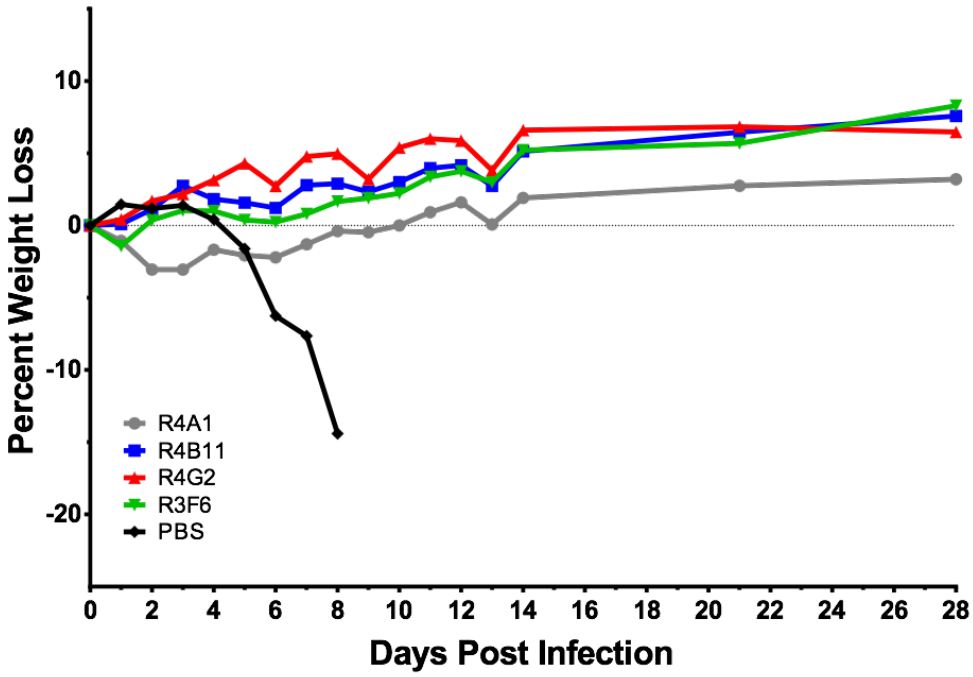
Supplemental Figure 2: Pseudovirion Neutralization Assay



Supplemental Figure 3: Weight loss of IFNAR-/- mice administered MARV GP specific antibodies. Each mouse (n=20 per antibody group) was administered 100 ug of antibody the indicated treatment, irrelevant antibody (n=10), or PBS (n=10) on Days -1, 1, and 3. Mice were challenged with 1000 pfu MARV Ci67 on D0



Supplemental Figure 4: Percent survival following the re-challenge of IFNAR-/- mice administered MARV GP specific antibodies. Each mouse (n=10 per antibody group or n=5 for the PBS control) was rechallenged with 1000 pfu MARV Ci67 on D35 of the original study, D0 indicated above.



Supplemental Figure 5: Weight loss following the re-challenge of IFNAR-/- mice administered MARV GP specific antibodies. Each mouse (n=10 per antibody group or n=5 for the PBS control) was rechallenged with 1000 pfu MARV Ci67 on D35 of the original study, D0 indicated above.

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